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Development and validity assessment of ELISA test with recombinant chimeric protein of SARS-CoV-2

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ABSTRACT

Serological tests developed for COVID-19 diagnostic are based on antibodies specific for SARS-CoV-2 antigens. Most of the antigens consist of a fragment or a whole amino acid sequence of the nucleocapsid or spike proteins. We evaluated a chimeric recombinant protein as an antigen in an ELISA test, using the most conserved and hydrophilic portions of the S1-subunit of the S and Nucleocapsid (N) proteins. These proteins, individually, indicated a suitable sensitivity of 93.6 and 100% and a specificity of 94.5 and 91.3%, respectively. However, our study with the chimera containing S1 and N proteins of SARS-CoV-2 suggested that the recombinant protein could better balance both the sensitivity (95.7%) and the specificity (95.5%) of the serological assay when comparing with the ELISA test using the antigens N and S1, individually. Accordingly, the chimera showed a high area under the ROC curve of 0.98 (CI 95% 0.958–1). Thus, our chimeric approach could be used to assess the natural exposure against SARS-CoV-2 virus over time, however, other tests will be necessary to better understand the behaviour of the chimera in samples from people with different vaccination doses and/or infected with different variants of the virus.

1. Introduction

The emergence of diseases from the novel coronavirus 2019 (COVID-19) is a major health threat. The SARS-CoV-2 has caused >605 million cases and >6.5 million deaths, according to the WHO report from 31 August 2022 (WHO, 2022). The current method of detection involves the quantitative polymerase chain reaction (qPCR), which identifies viral nucleic acid in samples collected during the viraemic phase. A wide range of immuno-serological assays have been developed, using specific proteins of the virus, complementing molecular assays for the diagnosis of COVID-19 and making possible the assessment of the protection rate of the various vaccines against SARS-CoV-2 viruses over time (Vashist, 2020).

The single and positive-stranded RNA genome of the SARS-CoV-2 encodes for a non-structural polyprotein and structural proteins, including the spike (S) and nucleocapsid (N) proteins. The spike or S protein, constituted by the S1 and S2 subunits, is a trimeric glycoprotein that mediates receptor recognition, cell attachment, and fusion during

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viral infection. The S1 subunit participates in the recognition of the receptor ACE2 of the host cells through the region called receptor binding domain (S-RBD). The S2 subunit contributes to the viral fusion and entry into the host cell, changing the conformation of the cell membrane. The Nucleocapsid or N protein has the fundamental function of packaging the viral genome into a ribonucleoprotein particle and participates in the assembly of the virion through its interaction with the viral genome and membrane protein (Du et al., 2009; Premkumar et al., 2020; Huang et al., 2020).

Previous studies showed the effectiveness of the ELISA test in the detection of IgM and IgG antibodies against the SARS-CoV-2, using specific proteins of the virus as antigens. Xiang et al. (2020) standardized an ELISA based on the recombinant nucleocapsid protein of the virus. In patients confirmed with COVID-19, sensitivity, specificity, positive (PPV) and negative predictive value (NPV) and consistency rate of IgM were 77.3, 100, 100, 80.0 and 88.1%, respectively, and those of IgG were 83.3 (55/66), 95.0, 94.8, 83.8 and 88.9%. In patients with suspected COVID-19, IgM sensitivity, specificity, PPV, NPV and consistency were 87.5 (21/24), 100, 100, 95.2 and 96.4%, respectively, and those for IgG were 70.8 (17/24), 96.6, 85.0, 89.1 and 88.1% (Xiang et al., 2020).

Currently, the diagnostic test RT-qPCR is being performed only in patients in the viraemic phase and with symptoms compatible with COVID-19. The lateral flow assay-based rapid diagnostic test is a complementary technique which can identify new cases of covid-19 through the analysis of specific biomarkers of the virus such as nucleic acid, antibodies, and antigens (Kevadiya et al., 2021). The ELISA represents a tool of mass testing allowing analysis at a scale greater than that used by the lateral flow assays (individual analysis), providing a better estimation of the infection rate and the progress and/or control of the pandemic. In addition, ELISA can impact the flow of testing by enabling faster diagnosis and better monitoring of individuals infected with SARS-CoV-2.

Here, we proposed an ELISA test using a chimeric recombinant protein, containing the S1 and N proteins of SARS-CoV-2. With this tool we aim to complement molecular biology tests and other serological tests, making possible: (i) to increase the ability to evaluate SARS-CoV-2 circulation in the population ensuring a greater diagnostic coverage of the disease and subsequent control measures; (ii) to perform epidemiological studies, including remote areas, owing to the ease of the assay and low-cost equipment; (iii) to test asymptomatic population or those with mild symptoms (including healthcare professionals), and, especially, to assess both the natural exposure and the protection rate of the various vaccines against SARS-CoV-2 virus over time.

2. Materials and methods

Human sera samples collection. The rapid lateral flow serological test (DPP® Covid-19, Biomanguinhos-Fiocruz) was used to evaluate IgM and IgG antibodies against SARS-CoV-2 on volunteers who agreed to participate in the research, during the pandemic in the second half of 2020 and January 2021. Of the total samples collected, 68 sera showed detectable IgG with positive RT-qPCR results. The time elapsed between the appearance of symptoms and the antibodies detection was, on average, five months. A total of 78 sera revealed non-reactive IgG with negative RT-qPCR results. Additionally, 30 pre-pandemic sera with negative IgG, provided by LACEN-MS from a sample bank collected between 2017 and 2019, were included in the analyses.

The rapid lateral flow serological test (DPP® Covid-19, Biomanguinhos-Fiocruz) was used as the gold standard test for all comparisons performed between the antigens and each of the different condition of samples. It is an immunochromatographic test that allows the detection of IgM and IgG antibodies in the same sample in two independent reactions, simultaneously. In addition, the platform makes possible the electronic reading of the result using a Micro-DPP Reader, eliminating the subjectivity of reading and reducing the possibility of human error. However, it is a test that only allows the analysis of one sample at a time.

ELISAs Tests. The SARS-CoV-2 proteins, Spike protein (S), S1-subunit of S protein, S2-subunit of S protein, the receptor-binding domain (S-RBD) and the nucleocapsid protein (N) were tested individually with each of the collected sera using the indirect ELISA. All tests with sera and proteins were performed by the same operator.

The optimal concentrations of proteins and sera were standardized after testing serial dilutions of both. The antigens were diluted in base two starting with an initial concentration of 0.5 μg/mL. The sera were tested in 3 serial dilutions: 1/25, 1/50 and 1/100. The results of the absorbance readings were plotted to define the ideal concentrations. Analysis of these curves and the ratio of absorbance in serum positive/negative allowed the choice of dilutions of the antigens and sera.

To carry out tests with each virus protein, polystyrene 96-well plates (Costar 3590 - Merck, USA) were adsorbed with each antigen in a carbonate-bicarbonate buffer, pH 9.6, for 2 h at 37 °C. The plates were then blocked with 100 μL/well of phosphate-buffered saline with 0.1% Tween 20 (PBST) and 5% skim milk for 60 min at 37 °C. After five washes with PBST, 100 μL/well of the sera, diluted 1:25 in PBST with 2% skim milk, were added and incubated for 60 min at 37 °C. Then, 100 μL of human anti-IgG (γ-chain specific) - peroxidase antibody produced in rabbit (SAB3701294 – Sigma, USA) (diluted in PBST) were added to each well, after the plates were washed five times with PBST. The plates were incubated for 30 min at 37 °C, washed five times, and 50 μL/well of substrate tetramethylbenzidine-TMB- Sigma-Aldrich (Goka and Farthing, 1987) was added. The reactions were stopped with 2.5 N H_2SO_4, and the results were read in an ELISA reader with a filter of 450 nm.

Development of the chimera. The synthetic chimeric protein was constructed using the DNAstar software (www.dnastar.com). Conserved sequences of the hydrophilic domains of the S1 and nucleocapsid (N) proteins, which showed better results with the ELISA test, were selected from the strain Wuhan-Hu-1 (Accession NC_045512) of SARS-CoV-2. The synthesis, purification and expression of the protein was performed by GenOne Biotechnology Solutions (https://www.genone.com.br/), using the *Escherichia coli* BL21(DE3) strain. Bacteria were grown in LB medium containing 100 mg liter^{-1} ampicillin. The induction was performed with 0.5 mM IPTG (isopropyl-β-D-thiogalactopyranoside) at 37 °C. The cells were lysed by ultra-sonication (Φ3, 3 s on/6 s off, 5 min) in lysis buffer (20 mM Tris-HCl, 300 mM NaCl, pH 8.0). The target protein was purified by Ni NTA Beads 6FF after gene expression.

The protein was analyzed by SDS-PAGE and Western Blot and the quantity and purity obtained was 1.5 mg and 85%, respectively.

The performance of the recombinant ELISA was compared with the commercial anti-SARS-CoV-2 (IgG) ELISA kit from the company EUROIMMUN, following the recommended protocols and using the same panel of sera.

Statistical analysis. The performance of the different individualised viral proteins and their chimera constitution were compared with the gold standards, in order to calculate sensitivity and specificity. The cut off point was calculated using the Receiver Operating Characteristic (ROC) curves with a confidence interval (CI) (95%) for the area under the ROC curve (AUC) and significance statistical level $\alpha = 0.05$.

The curves were drawn for each antigen/protein analyzed using the software Epitools (https://epitools.ausvet.com.au/roccurves). The ROC analysis is a powerful tool to measure the variation in sensitivity and specificity of the antigens tested by ELISA under specific conditions. The closer the ROC curve approaches the upper left corner, the better the quality of the test in terms of its ability to discriminate between groups. The diagonal reference line of the ROC curve represents a region of complete randomness of the test, which does not differentiate between sick and healthy patients.

AUC is an effective way to summarise the overall diagnostic accuracy of the test and was interpreted following Mandrekar (2010). It takes values from 0 to 1, where a value of 0 indicates a perfectly inaccurate

test and a value of 1 reflects a perfectly accurate test. In general, an AUC of 0.5 suggests no discrimination (i.e., ability to diagnose patients with and without the disease or condition based on the test), 0.7 to 0.8 is considered acceptable, 0.8 to 0.9 is considered excellent, and >0.9 is considered outstanding.

The Youden Index (J) was used to evaluate the maximum potential effectiveness of the biomarker (the virus protein). The index is also considered the main summary statistic of the ROC curve. Based on this index, we defined the optimal cut-point that achieves this maximum, optimising the biomarker's differentiating ability when the sensitivity and the specificity have equal weight. Thus, based on the ROC curve analyses and *J*, we dichotomized the quantitative values obtained from the ELISA results of the individualised proteins or their chimeric form.

Finally, we used the McNemar's Test to check the marginal homogeneity hypothesis of two paired tests. The analysis was performed using the Epitools software (Sergeant, 2018).

3. Results

The evaluation of the ELISA test was performed with each protein of the virus, individually, using the sera with reagent/non-reagent IgG results, according to the rapid serological test. The optimal dilutions of the conjugate and the concentrations of use of each antigen are shown in Table 1. The most favourable dilutions of the sera were 1:25 for all the antigens in the ELISA test.

A total of 40 sera were tested with the S1 protein, while the other proteins (S-RBD, S, S2 and N) were tested with more samples (146, 135, 118 and 117, respectively; Table 1). For this reason, the comparisons in which the protein S1 was involved may have been partially impaired by its relatively small N.

The results were expressed as the relative sensitivity or specificity of each antigen in the ELISA test considering the serological rapid test as the reference for the analysis.

Fig. 1 shows the ROC curves obtained with each protein. ELISA tests using S1 and N proteins separately showed the highest AUC values. Similarly, the sensitivity and specificity values of the ELISA using S1 and N proteins, separately, were the highest values compared to other proteins (Table 2). For these reasons, the S1 and N proteins were selected to constitute the chimeric protein.

The S1/N chimera was tested by the ELISA technique (using the optimal dilution of 1:50,000 of the conjugate and the final concentration of 2 μg/ml of the antigen) to evaluate and compare the detection capability of the IgG antibodies in sera tested previously with each individual antigen.

When comparing the results among the ELISA tests with each antigen (S1/N chimera, S1, S-RBD and N) and the rapid serological test (gold standard), using the McNemar test, no significant differences were observed (*p* values of >0.99, 0.56, 0.17, and > 0.99, respectively). On the other hand, the ELISA tests using the antigens S and S2 showed significant differences compared with the serological rapid test ($p = 0.00007629$ and $p = 0.04904$, respectively). Table 2 presents the cut-off points, Sensibility, Specificity, AUC and Younden's J statistical values for proteins that did not show differences in relation to the rapid test.

Table 1
Number of sera tested by ELISA test with each protein of SARS-CoV-2, optimal dilutions of conjugate and proteins (S protein, S1 protein, S2 protein, S-RBD protein and N protein).

ELISA	Conjugate	Recombinant proteins (final concentration μg/mL)	Number of sera tested with each protein
S1	1:80,000	2.0	40
S-RBD	1:50,000	2.0	146
S	1:50,000	2.0	135
S2	1:50,000	1.0	118
Nucleocapsid	1:50,000	1.0	117

From the AUC and Youden's J statistical results, apparently the S1/N chimera had a similar performance to that obtained with S1 and slightly superior to that of the N and S-RBD proteins. Additionally, the chimera presented the most balanced sensitivity and specificity values when compared to the performance of S1 and N proteins separately. Fig. 2 shows the ROC analysis and the AUC result using the chimeric protein S1/N for the ELISA and having the serological rapid test as "gold standard".

The S1/N chimera (as the gold standard test with cut-off point 0.315) showed no significant differences by the McNemar test when comparing with the results of the proteins S1, N, and S-RBD (*p*-values 0.50, 0.50, and 0.38, respectively). On the other hand, the results of S (cut-off point 0.244) and S2 (cut-off point 0.246) proteins were significantly different from the S1/N chimera (McNemar *p* values of 0.0075 and borderline 0.07). The chance of false negative results was significantly higher than that of false positive when using protein S (OR = 5, 95%CI 1.44–17.27). This same trend was borderline when the S2 protein was used. Table 3 presents the cut-off points, sensitivity, specificity, AUC and Younden's J statistical values for proteins that did not show differences in relation to the S1/N chimera.

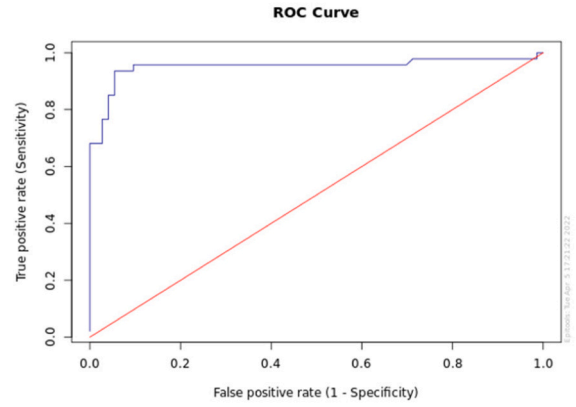
In addition, using the Anti-SARS-CoV-2 (IgG) ELISA (Euroimmun) as the reference test, the sensitivity and specificity of the S1/N chimera ELISA were 96,4% (95% CL: 82.3–99.4%) and 89,8% (95% CL: 79.5–95.3%), respectively. No significant differences were detected between both tests (McNemar's test *p*-value = 0.12,), and the Chimera Elisa showed a high AUC value (Fig. 3). However, the ELISA test using the N protein alone was significantly different from the commercial test (McNemar test p-value = 0.0019), and the S1 protein could not have been compared with the commercial test in this study because it was denatured.

4. Discussion

We developed the chimeric recombinant protein, containing S1 and N proteins of SARS-CoV-2, which was used as an ELISA test to detect exposures to this pandemic virus. Such ELISA test showed outstanding AUC, and high sensitivity and specificity values, with no significant differences relative to the rapid test (gold standard) and a commercial test (one more reference). The nucleotide sequences of the genes coding the S1 and Nucleocapsid proteins were searched in GenBlast to obtain the amino acid sequences of each one. A Blast was performed between both proteins to obtain the most conserved and hydrophilic portions of S1 and N proteins, using the DNAstar software.

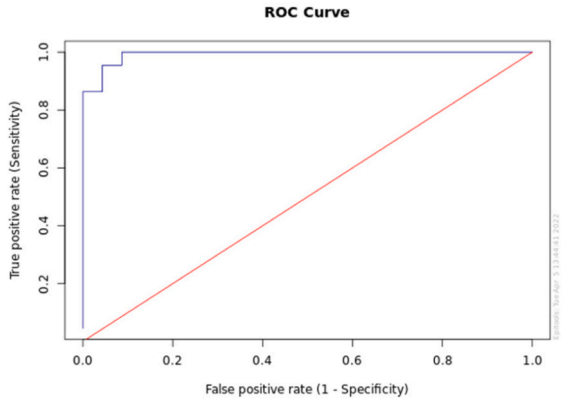
Our results are important because new tests for diagnostics are still useful as the WHO currently still considers COVID-19 a global emergency. Thus, the accurate diagnosis of COVID-19 in infected people is fundamental to reduce the spread of the virus and perform proper treatment in the early stages of the disease. To date, the quantitative reverse-transcriptase polymerase chain reaction (RT-qPCR) is the most widely employed method of detection of SARS-CoV-2, identifying viral nucleic acid in samples collected during the viraemic phase. The plaque-reduction neutralisation assays are also considered reference standard tests because they allow assessing the presence of antibodies that inhibit infection in culture cells, however, they are time-consuming, restricted to biosafety level 3 laboratories, difficult to standardise and difficult to implement on a large scale (Dolscheid-Pommerich et al., 2022). These limitations favor the use of ELISA based on proteins of the SARS-CoV-2 as antigens to detect IgM and IgG antibodies against the virus (Krähling et al., 2021).

Previous studies have shown that the specific antibody kinetics may be determined by the target antigen (Chen et al., 2020; Liu et al., 2020; Jalkanen et al., 2021; Semmler et al., 2021). The viral surface spike glycoprotein (S) and the nucleocapsid protein (N) appear to be the main targets of SARS-COV-2-specific antibodies (predominantly IgG) in both the acute and post infection phases. The S protein of SARS-CoV-2 is a major immunogenic protein, and the N protein is another target for most



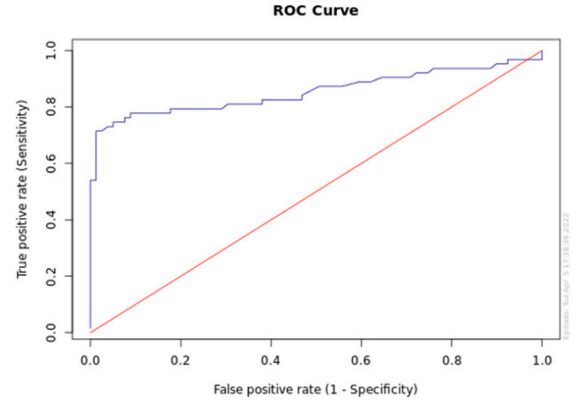
N protein
AUC= 0.951(CI 95% 0.9-1.0)

A



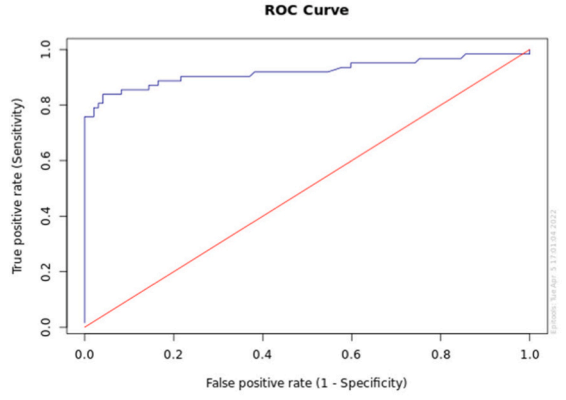
S1 protein
AUC= 0.992 (CI 95% 0.99-0.977)

B



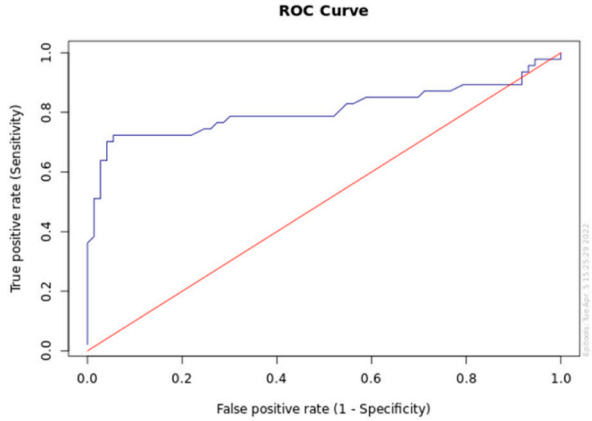
S protein
AUC= 0.854 (CI 95% 0.78-0.92)

C



S-RBD protein
AUC= 0.921 (CI 95% 0.867-0.975)

D



S2 protein
AUC= 0.807 (CI 95% 0.71-0.90)

E

Fig. 1. ROC analysis of ELISA tests with each antigen: A- N protein, B- S1 protein, C- S protein, D- S-RBD protein, *E*- S2 protein. Area Under the ROC Curve (AUC), confidence interval (CI).

Table 2
Cut-off points, Sensibility, Specificity, AUC and Younden's J statistical values for S1, N, S-RBD and S1/N chimera having the rapid serological test as the gold standard for the analysis.

Protein	Cut-off points	Sensitivity	Specificity	AUC (IC 95%)	Younden's J statistic
S1/N chimera	0.315	0.957	0.955	0.98 (0.958–1)	0.912
S1	0.264	1.00	0.913	0.99 (0.977–1)	0.913
N	0.246	0.936	0.945	0.951 (0.9–1)	0.881
S-RBD	0.288	0.839	0.959	0.921 (0.867–0.975)	0.798

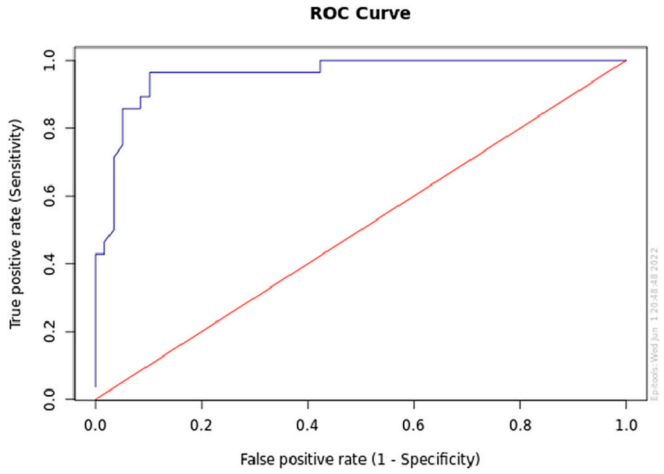


Fig. 2. ROC analysis using S1/N chimeric protein antigen for the ELISA test using the serological rapid test as reference. Area under ROC curve (AUC), confidence interval (CI).

Table 3
Cut-off points, Sensitivity, Specificity, AUC and Younden's J statistical values for each S1, N and S-RBD having the S1/N chimera as the gold standard test (cut-off point 0.315).

Protein	Cut-off points	Sensitivity	Specificity	AUC (95%CI)	Younden's J statistic
S1	0.285	0.957	1.00	0.997 (0.99–1.00)	0.957
N	0.274	0.935	0.972	0.976 (0.944–1.00)	0.907
S-RBD	0.2	0.873	0.951	0.945 (0.901–0.989)	0.824

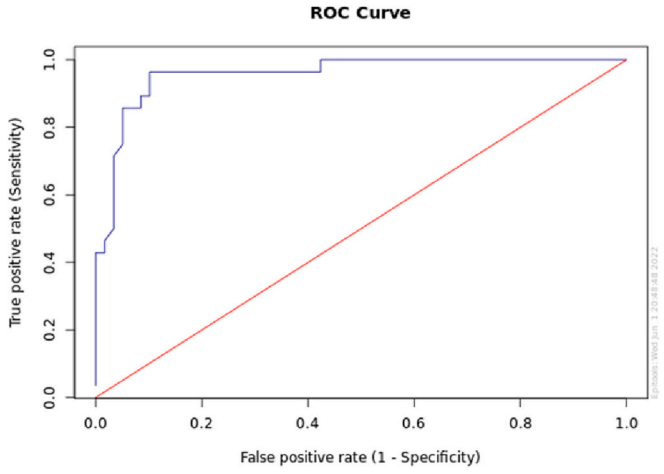


Fig. 3. ROC analysis with Anti-SARS-CoV-2 (IgG) ELISA (Euroimmun). Area under ROC curve (AUC), confidence interval (CI).

serological assays for coronavirus (CoVs) because of its abundant expression. The S protein has been used in IFAs, ELISAs and WB analyses, and in general, antibodies directed against this protein appeared later in infection (Meyer et al., 2014), and the N protein has been widely used in recombinant serological assays (Qiu et al., 2005).

Chia et al. (2020), using a capture ELISA technique, found that the S-RBD protein provides high specificity, when compared with S1 and N proteins. On the other hand, our results using the ELISAs with N and S1 proteins, individually, indicated a suitable and higher sensitivity (93.6% and 100%) and specificity (94.5% and 91.3%), respectively, for IgG antibody detection, than those specificity (95.1%) and a sensitivity (87.3%) found for S-RBD protein. Additionally, the ELISA using the three antigens was not considered different in detecting SARS-COV-2-specific antibodies having the serological rapid test as gold standard.

According to our results, some authors mentioned higher sensitivity of the N protein with respect to the S protein (Fenwick et al., 2021; Kohmer et al., 2020; Stringhini et al., 2020). Therefore, we used the N protein for the design of the chimera. The ELISA test using the S1/N chimera of the SARS-CoV-2 showed more balanced values of sensitivity (95.7%) and specificity (95.5%) when compared to those obtained with the antigens N and S1, individually (sensitivity 1.00 and 0.91 and specificity 0.91 and 0.94 for S1 and N, respectively). These findings are promising when thinking about the constitution of the chimeric protein and the improvement of the ELISA serological test for diagnosing in the population.

In conclusion, we have tested proteins of SARS-CoV-2 virus (S1, S2, S, S-RBD and N) on sera samples against IgG SARS-COV-2-specific antibodies. We obtained higher sensitivity and specificity with the S1 and N proteins and, in consequence, the most conserved and hydrophilic portions of both proteins were used in the design and construction of the chimera. The S1/N chimeric protein improved the balance between the sensitivity and the specificity of the ELISA test when comparing with the antigens N and S1, individually. Thus, the ELISA test proposed with the S1/N chimera represents an alternative for the COVID-19 diagnostic, however, other tests will be necessary to better understand the behaviour of the chimera in samples from people with different vaccination doses and/or infected with different variants of the virus.

Ethical approval

This study was approved by the Fiocruz-Brasilia Ethics Committee (CAAE 37183920.9.0000.8027).

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